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CpG-ODN-induced nitric oxide production is mediated through clathrin-dependent endocytosis, endosomal maturation, and activation of PKC, MEK1/2 and p38 MAPK, and NF-κB pathways in avian macrophage cells (HD11)

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Abstract

We have characterized the nitric oxide (NO) induction by CpG oligodeoxydinucleotide (CpG-ODN) and lipopolysaccharide (LPS) in an avian macrophage cell line (HD11) and evaluated signal transduction pathways by using selective inhibitors. Our results indicate that while CpG-ODN and LPS both stimulate inducible NO synthase (iNOS) to produce NO through common signalling pathways involving activation of protein kinase C (PKC), mitogen-activated protein kinases (p38 MAPK and MEK1/2) and transcription factor NF-κB; CpG-ODN inducing NO production distinctively requires a clathrin-dependent endocytosis and subsequent endosomal maturation. Inhibitors of clathrin-dependent endocytosis such as monodansylcadaverine and hyperosmolar sucrose completely abolished CpG-ODN stimulated NO production by HD11 cells, but have no or less effect on LPS-induced NO production. The endosomal maturation is also critical for stimulation of NO induction by CpG-ODN, but not by LPS. Our findings are the first to demonstrate cellular signalling pathways that mediate CpG-ODN immunostimulatory activity in cells from non-mammalian species.
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Keywords: CpG-ODN; NO; PKC; MAPK; NF-κB; Clathrin; Endocytosis; Avian

1. Introduction

Nitric oxide (NO) is a multi-functional mediator with diverse physiological and pathological roles in, for example, vasodilatation, neurotransmission, and host defence against infectious agents and tumours [1–4]. There are three distinct isoforms of nitric oxide synthase: neuronal (nNOS or NOS-1), inducible (iNOS or NOS-2) and endothelial (eNOS or NOS-3) [5]. The constitutive isoforms, eNOS and nNOS, found in endothelial cells and neurons produce small quantity of NO that acts primarily as a cell signalling molecule [2,3]. The inducible isoform found in the macrophage cells produces large amount of NO when cells are exposed to intracellular pathogens, certain tumour cells, microbial products such as lipopolysaccharide (LPS), and cytokine interferon-γ (IFN-γ) [4]. Production of NO by activated macrophages is an important innate immune response, critical for the bactericidal activity of macrophages [6].

DNA from bacteria, but not vertebrates, is a powerful stimulator of the innate immune system. The immunostimulatory activities of bacterial DNA are attributed to the presence of high frequency of unmethylated CpG dinucleotide [7]. It has been well documented that bacterial DNA and synthetic oligodeoxynucleotide containing unmethylated CpG-dinucleotides (CpG-ODN) activate B-lymphocyte and innate immune cells (macrophage, dendritic cells, and natural killer cells) to secrete cytokines [interleukin-1β (IL-1β), IL-6, IL-12, IL-18, tumour necrosis factor-α (TNF-α), interferon-α (IFN-α), and IFN-γ] and to promote the acquired immunity [8–19]. Bacterial DNA and CpG-ODN also induce NO production in murine and bovine macrophages [20–22].

Abbreviations: ANOV, analysis of variance; CpG-ODN, CpG oligodeoxydinucleotide; DMEM, Dulbecco's modified eagles media; IL-1β, interleukin-1β; ILR/TLR, IL-1 receptor/Toll-like receptor; iNOS, inducible nitric oxide synthase; IFN-γ, interferon-γ; LPS, lipopolysaccharide; LBP, LPS binding protein; MAPK, mitogen-activated protein kinase; MEK1/2, MAPK kinase; MDC, monodansylcadaverine; NF-κB, nuclear factor-κB; PKC, protein kinase C; TNF-α, tumor necrosis factor-α.

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However, mechanisms involved in the NO induction by bacterial DNA or CpG-ODN are not well characterized. Some of these studies indicated that priming macrophages with IFN-γ or the presence of a subthreshhold concentration of lipopolysaccharide (LPS) were required in order for bacterial DNA or CpG-ODN to induce NO production [20,21]. Only a recent study showed that CpG-ODN by itself could stimulate NO production in the murine macrophage cell line (RAW 264.7) [23].

CpG-ODN and LPS share certain common signal transduction pathways. For example, both CpG-ODN and LPS engage signal transduction via IL-1 receptor/Toll-like receptor (ILR/TLR) family [24]. However, LPS requires LPS-binding protein (LBP) and CD14 to engage the cell surface [25] or intracellular (Golgi apparatus) [26,27] located TLR4 via a CD14-dependent internalization of LPS by macropinocytic pathway [28]. CpG-ODN interacting with its receptor, TLR9, occurs exclusively in the intracellular compartment [25]. Internalization by endocytosis as well as endosomal maturation is therefore the precondition for CpG-ODN activity [29,30].

There is very little information regarding stimulatory activity of CpG-DNA in avian immune cells. Our previous study confirmed CpG motif-dependent induction of NO production in an avian macrophage cell line (HD11) by synthetic CpG-ODN. The GTCGTT was found to be the most active CpG motif to stimulate HD11 cell responses including IL-1β expression and NO production [31]. In this study, we further evaluated the pathway of signal transduction in CpG-ODN-induced NO production in HD11 cells. Due to lack of appropriate avian-specific antibodies to proteins or enzymes involved in signal transduction cascades, selected pharmacological inhibitors that have proved to be specific for various signalling steps were used in this study. LPS, a well-characterized microbial molecule, was used for the comparison. Our results demonstrated that induction of NO production in HD11 cells by CpG-ODN requires clathrin-dependent endocytosis, endosomal maturation, and downstreaming signal transduction involving protein kinase C (PKC), MAPK, and NF-κB activation.

2. Materials and methods

2.1. Materials

Synthetic ODNs were purchased from BioSource International (Camarillo, CA, USA) and further purified by ethanol precipitation. The sequences of synthetic ODNs used in the present study were: CpG-ODN, GTC GTT GTC GTT GTC GTT and a control ODN without CpG motif (nCpG-ODN), CCA TGG CCA TGG CCA TGG. Clathrin-dependent endocytosis inhibitor, monodansylcadaverine (MDC), and endosomal maturation inhibitor, chloroquine, were purchased from Sigma (St. Louis, MO, USA). PKC inhibitor (Ro 32-0432), p38 MAPK inhibitor (SB-203580), MAPK kinase (MEK) inhibitor (U-0126), IκB phosphorylation inhibitor (Bay 11-7086), and iNO inhibitor (1400W) were purchased from Biomol (Plymouth Meeting, PA, USA). All media and medium-additives were purchased from Sigma. Cell culture 96-well plate was purchased from Becton Dickinson (Franklin Lakes, NJ, USA).

2.2. Cell culture

The HD11, an avian macrophage cell line (gift from Dr. K Klasing, UC, Davis, CA, USA), was maintained in the Dulbecco's modified eagles media (DMEM) containing 10% chicken serum, antibiotics (100 U penicillin/ml and 100 μg streptomycin/ml), and 1.5 mM L-glutamine at 41 °C, 5% CO_2, and 95% humidity incubator. For cell inhibition and stimulation assay, a 100-μl aliquot of 2×10^6 HD-11/ml was seeded to each well of a round-bottomed 96-well plate and allowed to grow overnight (16–18 h). The medium was replaced with fresh medium containing appropriate inhibitors and pre-incubated for 1 h followed by stimulation with CpG-ODN or LPS for additional 24 h in a final volume of 200 μl/well.

2.3. Inhibition of clathrin-dependent endocytosis and endosomal maturation

HD11 cells were pretreated with various concentrations of MDC for 1 h and then CpG-ODN (2.5 μg/ml) or LPS (1 μg/ml) was added to the medium. Cells were then cultured for an additional 24 h. For hyperosmolar sucrose inhibition assay, cell culture media was replaced with hypertonic medium containing 0.45 M sucrose. Then either CpG-ODN (2.5 μg/ml) or LPS (1 μg/ml) was added to the appropriate wells immediately and the cultures were incubated for 30 min. After 30-min incubation, medium was removed, cells were washed twice with medium, and the plate was replenished with 200-μl fresh medium per well with no agonists. Cells were cultured for an additional 24 h. To inhibit the endosomal maturation, cells were pretreated with various concentrations of chloroquine for 1 h and then stimulated with either CpG-ODN or LPS for 24 h.

2.4. Inhibition of signal transduction

HD11 cells were pretreated with various concentrations of PKC inhibitor (Ro 32-0432), p38 MAPK inhibitor (SB-203580), MAPK kinase (MEK) inhibitor (U-0126), IκB phosphorylation inhibitor (Bay 11-7086), and iNOS inhibitor (1400W) for 1 h and then either CpG-ODN (2.5 μg/ml) or LPS (1 μg/ml) was added to the medium and cells were cultures for an additional 24 h.

2.5. Nitrite production assay

Nitrite production by activated HD11 was measured by the Greiss assay [32]. Briefly, aliquot of 100 μl culture superna-

tant from each well was transferred to a new flat-bottom plate 96-well plate and combined with 50-μl of 1% sulfanilamide and 50 μl of 0.1% naphthylenediamine (both were prepared in 2.5% phosphoric acid solution). After 10-min incubation at room temperature, the nitrite concentration was determined by measuring optical density (OD_{595}) of each well using a SPECTRA MAX microplate reader (Molecular Devices, Sunnyvale, CA, USA). Sodium nitrite (Sigma) was used as a standard. Nitrite content in the cell-free medium was subtracted from the value obtained with cells.

2.6. Data analysis

Data are means and standard deviations of three independent experiments with three to five replicates each. Statistical difference was determined at the level of $p < 0.05$ by Student's *t*-test using the SigmaStat software (Jandel, San Rafael, CA, USA).

3. Results

3.1. Characterization of CpG-ODN- and LPS-stimulated nitrite production in HD11

Synthetic CpG-ODN containing the GTCGTT motif is immunostimulatory and, by itself, a powerful stimulant of NO production in HD11 cells. CpG-ODN activated HD11 cells and induced dose-dependent NO production. Phosphorothioate CpG-ODN was shown here to be a more potent stimulant than the phosphodiester form (Fig. 1A). Maximal stimulation was obtained when cells were stimulated with 1.25–2.5 μg phosphorothioate CpG-ODN/ml. The concentration for maximal induction of NO in HD11 cells by LPS was estimated at 0.64 μg/ml (Fig. 1B). There was no synergistic or additive effect on NO production when cells were stimulated simultaneously with both CpG-ODN and LPS (Fig. 1B). The exposure time for maximum stimulation of NO production with both CpG-ODN and LPS was determined as following: HD11 cells were pulsed with CpG-ODN (2.5 μg/ml) or LPS (1 μg/ml) for different time intervals followed by washing the cells twice with media and continued culture for additional 24 h. Results indicated that a minimum of 4 h of exposure was required for the full activation of HD11 cells by either CpG-ODN or LPS (Fig. 1C). Both CpG-ODN and LPS induced NO production by iNOS, since these inductions can be inhibited by an iNOS-specific inhibitor 1400W (Fig. 1D).

3.2. Role of clathrin-dependent endocytosis and endosomal maturation in CpG-ODN- and LPS-induced NO production

CpG-ODN and LPS differentially engage their respective receptors, TLR9 and TLR4, to initiate signal transduction leading to activation of immune cells. It is apparent that CpG-ODN interacting with TLR9 occurs via a different pathway from LPS. The effects of two mechanistically distinct inhibitors of clathrin-dependent endocytosis, MDC

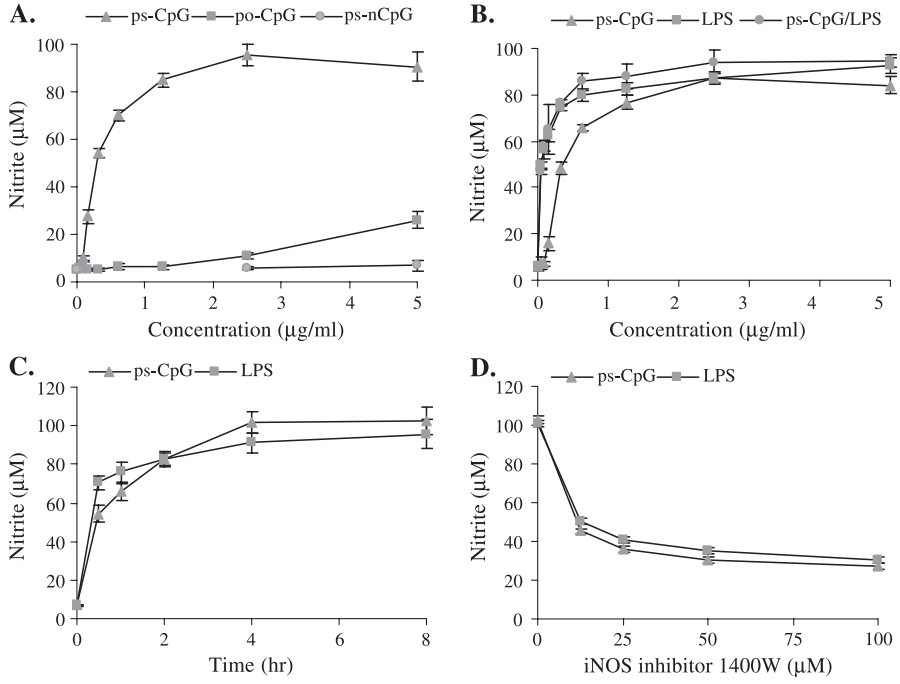

Fig. 1. Characterization of CpG-ODN- and LPS-stimulated NO production in avian macrophage cells (HD11). (A) Comparison of immunostimulatory activity of phosphorothioate CpG-ODN (ps-CpG), phosphodiester CpG-ODN (po-CpG), and a control ODN without CpG motif (ps-nCpG). (B) Comparison of stimulatory activity of CpG-ODN, LPS, and combination of CpG-ODN and LPS for NO production. (C) Duration of cell exposure required for CpG-ODN and LPS to stimulate maximal NO production. (D) Effect of iNOs inhibitor on CpG-ODN- and LPS-induced NO production.

and hypertonic media, on CpG-ODN- and LPS-induced NO production were evaluated. Pretreatment of cells with MDC resulted in dose-dependent inhibition of CpG-ODN-induced NO production; whereas, LPS-induced NO production was not affected (Fig. 2A). Cells treated with hypertonic media containing 0.45 M sucrose reduced both CpG-ODN- and

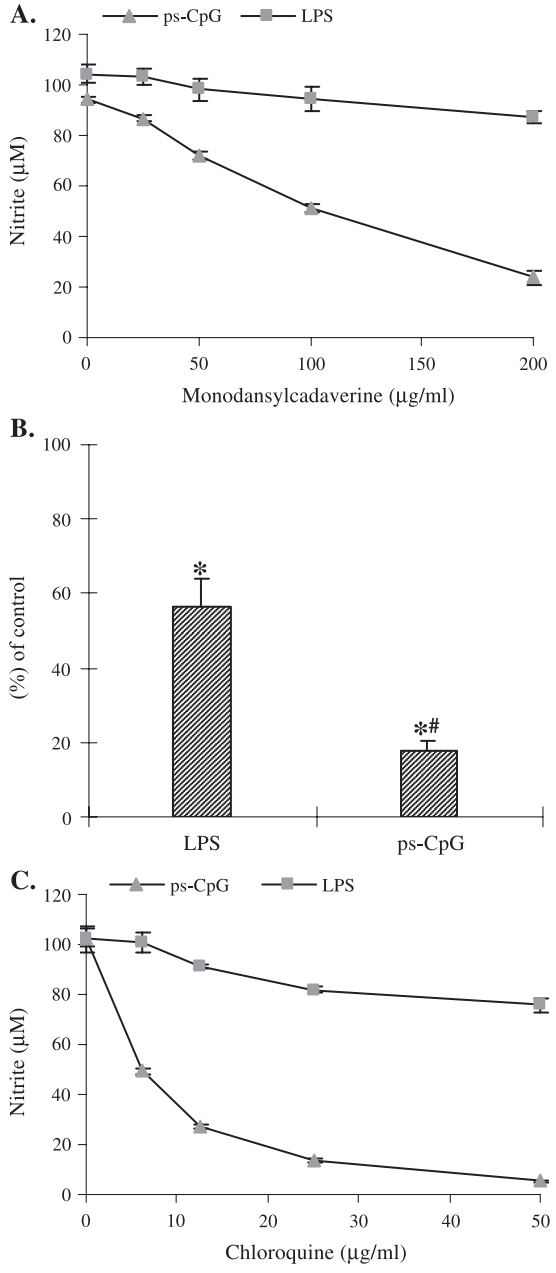

Fig. 2. Roles of clathrin-dependent endocytosis and endosomal maturation/acidification in CpG-ODN- and LPS-stimulated NO production. (A) Effect of clathrin-dependent endocytosis-specific inhibitor, MDC, on CpG-ODN- and LPS-stimulated NO production. (B) Effect of hypertonic medium (0.45 M sucrose) on CpG-ODN- and LPS-stimulated NO production. The sign * indicates difference ($p < 0.05$) compared to control in which cells were not treated with hypertonic medium and # indicates difference ($p < 0.05$) compared to LPS-stimulated cells. (C) Effect of endosomal maturation/acidification inhibitor, chloroquine, on CpG-ODN- and LPS-stimulated NO production.

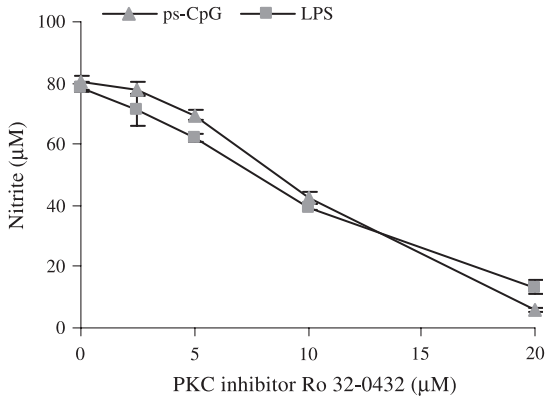

Fig. 3. Effect of inhibition of PKC by Ro 32-0432 on CpG-ODN- and LPS-stimulated NO production.

LPS-induced NO production. However, the reduction on CpG-ODN-induced NO production by hypertonic media was much greater than on LPS (Fig. 2B). These results are strong evidence indicating clathrin-dependent endocytic pathway is involved in CpG-ODN signal transduction. Our results further showed that endosomal maturation is critical for CpG-ODN-stimulated HD11 cell activation. Increasing concentration of chloroquine, an endosomal maturation inhibitor, in the media completely blocked the CpG-ODN-mediated induction of NO (Fig. 2C). In contrast, chloroquine has much less inhibitory effect on LPS-induced NO production.

3.3. Role of PKC in CpG-ODN- and LPS-induced NO production

The PKC family of isozymes catalyze numerous protein phosphorylations that are essential signalling steps for cellular activation and differentiation. The PKC family is

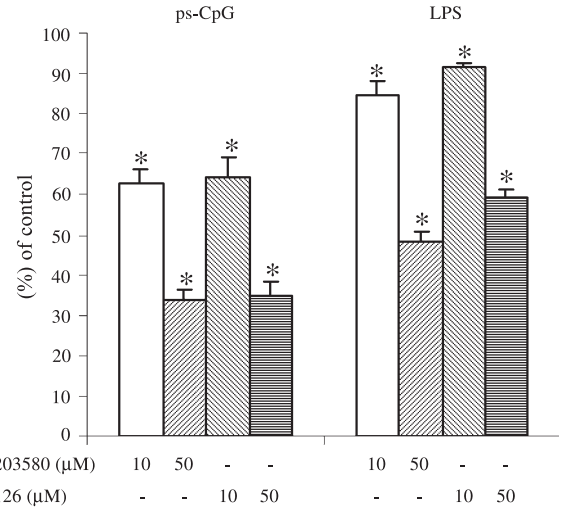

Fig. 4. Effects of MAPK inhibitors, SB-203580 and U-0126, on CpG-ODN- and LPS-stimulated NO production. The sign * indicates difference ($p < 0.05$) compared to the control in which cells were not pretreated with inhibitors.

classified into to sub-groups, Ca^{2+}-dependent PKC (-α, -β_I, -β_{II}, and -γ) and Ca^{2+}-independent PKC (-δ, -ξ, -ν, and -L). Ro 32-0432 is a bisindolylmaleimide compound that selectively inhibits Ca^{2+}-dependent PKC at its ATP binding site [33,34]. Ro 32-0432 inhibited both CpG-ODN- and LPS-induced NO production dose-dependently. The high concentration (20 μM) of Ro 32-0432 completely abolished both CpG-ODN- and LPS-induced NO production (Fig. 3).

3.4. Role of MAPK in CpG-ODN- and LPS-induced NO production

The MAPK cascades play important roles in linking a variety of extracellular signals to cellular events such as growth, differentiation, apoptosis and inflammatory response [35]. SB-203580, a member of a novel class of pyridinyl imidazoles, selectively inhibits the p38 MAPK [36]. SB-203580 inhibited both CpG-ODN- and LPS-stimulated NO production in a dose-dependent manner (Fig. 4). The compound U-0126 is a highly selective inhibitor of MAPK family members, MEK1/2 [37]. Incubation of cells with increasing concentrations of U-0126 diminished both CpG-ODN-and LPS-induced NO production (Fig. 4).

3.5. Role of NF-κB in CpG-ODN- and LPS-induced NO production

The transcription factors of NF-κB family remain in a quiescent state, complexed with inhibitory IκB proteins, in the cytosol of virtually all vertebrate cells [38]. Upon activation, IκB proteins are phosphorylated and released from the NF-κB that then undergoes nuclear translocation and initiation of gene transcription. BAY 11-7082 prevents the activation of NF-κB by inhibiting IκB phosphorylation [39,40]. Incubation of cells with BAY 11-7082 reduced both CpG-ODN- and LPS-induced NO production. High concentration of BAY 11-7082 in the media (50 μM) completely abolished the NO production in HD1 cells stimulated by both CpG-ODN and LPS (Fig. 5).

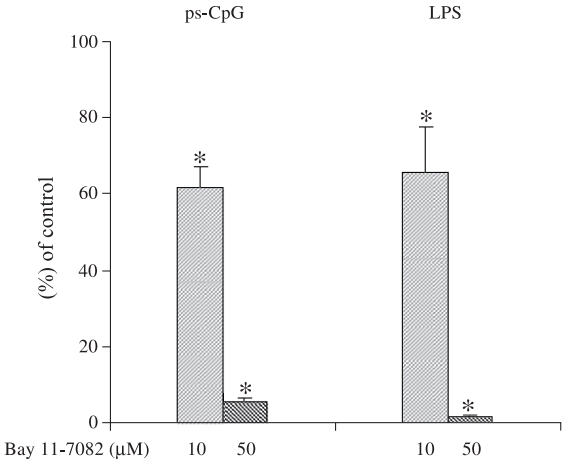

Fig. 5. Effect of blocking NF-κB activation by IκB phosphorylation inhibitor, Bay 11-7082, on CpG-ODN- and LPS-stimulated NO production. The sign * indicates difference ($p < 0.05$) compared to the control in which cells were not pretreated with inhibitor.

4. Discussion

Bacterial DNA or synthetic ODN containing CpG dinucleotides are immunostimulatory to most vertebrate species. These immunostimulatory activities of CpG-ODN have been studied extensively and are well characterized in human and murine immune cells. However, responses of avian immune system to bacterial DNA or CpG-ODN are largely unknown. Previous study in our lab has identified a CpG motif that is immunostimulatory to an avian macrophage cell line, inducing innate immune responses such as NO production and IL-1β expression [31]. In this study, the biochemical characteristics and the signal transduction pathway of NO production induced by this CpG-ODN were further evaluated.

In the present study, CpG-ODN with the GTCGTT motif has been demonstrated to be immunostimulatory and, by itself, a powerful stimulant of NO production in avian macrophage cell line HD11. Production of NO by macrophages after exposure to the microbial stimuli is an important bactericidal response of innate immune system and plays a critical role in the host defence against intracellular pathogen such as *Salmonella* [41]. Compared to other stimulatory activities, CpG-ODN-induced NO production in the macrophage cells has not been well characterized. Previous studies using a murine macrophage cell line suggested that priming the cell with IFN-γ or presence of minus amount of LPS is required for bacterial DNA or CpG-ODN to induce NO production [20,21]. In contrast, a recent study showed that murine macrophage cells are able to respond to CpG-ODN stimulation alone, resulting in induction of iNOS and NO production [23]. Results from the present study confirm that CpG-ODN-stimulated NO production in HD11 cells is CpG motif-dependent (Fig. 1A). CpG-ODN can stimulate NO production without prior cell activation or priming by other microbial stimuli. In contrast to the result reported previously [21], there was no synergistic effect on NO induction when cells were exposed to both CpG-ODN and LPS simultaneously (Fig. 1B). Furthermore, the characteristics of CpG-ODN- and LPS-induced NO production were also similar, which requires at least 4 h of exposure to the stimuli to obtain maximum NO production (Fig. 1C), and there was no apparent delay period in the CpG-ODN-induced NO production as previously reported [23]. In line with previous reported results [23], this study shows that both CpG-ODN and LPS stimulate NO production in HD11 cells through iNOS, since an iNOS specific inhibitor (1400W) at concentration of 100 μM blocked about 70% of the NO production (Fig. 1D).

CpG-ODN inducing NO production distinctively requires a clathrin-dependent endocytosis and subsequent

endosomal maturation. Inhibitors of clathrin-dependent endocytosis such as MDC and hyperosmolar sucrose abolished most of CpG-ODN-stimulated NO production in HD11 cells, but have less or no effect on LPS. The endosomal maturation is also critical for induction of NO by CpG-ODN, but not by LPS. CpG-ODN and LPS convey their immune stimulatory activities by engaging their respective receptors, TLR9 and TLR4. Due to differential cellular compartmentation of TLR4 and TLR9 [25,27], distinctive mechanisms are required for them to interact with LPS and CpG-ODN. LPS requires either LBP binding and subsequent complex with CD14, a membrane-anchored protein, before interacting with cell surface TLR4 [25] or the complex of LPS-CD14 has to be internalized via a macropinocytic pathway [28] in order to interact with intracellular (Golgi apparatus) located TLR4 [27,28]. The mechanism by which CpG-ODN engages its signal transduction through TLR9 is not yet defined. Studies have shown that TLR9 is exclusively intracellularly located [25] and cellular uptake by endocytosis and subsequent endosomal maturation are prerequisites for CpG-ODN signalling [29]. In this study, mechanistically distinctive clathrin-dependent endocytosis inhibitors, MDC (which inhibits clathrin-dependent endocytosis by stabilizing clathrin cages [42]) and hypertonic medium (which blocks clathrin association [43]), blocked 70–80% of CpG-ODN-induced NO production (Fig. 2A and C). These results, for the first time, demonstrated that CpG-ODN initiates signal transduction, which leads to cell activation and NO production in HD11, via a clathrin-dependent endocytic pathway. The finding of clathrin-dependent endocytosis mediated CpG-ODN signalling suggests possible involvement of receptor(s), since clathrin-dependent endocytosis generally functions in receptor-mediated endocytosis [44–47]. Receptors that facilitate the uptake of bacterial DNA or CpG-ODN remain to be determined. Unlike LPS, endosomal maturation/acidification following endocytosis is indispensable in bacterial DNA and CpG-ODN-mediated cellular signalling. Previous studies have shown that endosomal maturation/acidification is required for bacterial DNA and CpG-ODN-stimulated leukocyte activation [29,30]. This study has confirmed the essential role of endosomal maturation/acidification in CpG-ODN-mediated cellular signalling by using a specific inhibitor, chloroquine, that blocks endosomal acidification. Chloroquine inhibited CpG-ODN-induced NO production in avian macrophage cells (HD11) dose-dependently and completely abolished the effect of CpG-ODN at high concentration (Fig. 2C).

PKC activity is indispensable for both CpG-ODN- and LPS-induced NO production in HD11 cells. PKC plays important roles in various signal transduction cascades and cellular functions. There are numerous PKC isozymes with different cellular distribution patterns and distinctive roles in cellular signal transduction [48,49]. Previous studies showed a critical role of PKC isozymes in MAPK cascade, NF-κB activation, as well as phagocytosis in mammalian macrophages [50–52]. Other studies have shown direct involvement of PKC in iNOS induction by LPS [53,54]. In this study, an isotype-specific PKC inhibitor, Ro 32-0432 that preferentially inhibit PKCα, was able to block both CpG-ODN- and LPS-induced NO in HD11 cells almost completely (Fig. 3), indicating that PKCα plays a critical role in CpG-ODN- and LPS-induced NO production in the avian macrophages.

MAPK pathway also plays significant roles in both CpG-ODN- and LPS-induced NO production in HD11 cells. Inhibition of either p38 MAPK or MEK1/2 activation by respective inhibitors SB-203580 and U-0126 significantly reduced NO production in both CpG-ODN- and LPS-stimulated HD11 cells. MAPK are a family of serine/threonine protein kinases that participate in signalling pathways initiated by many extracellular stimuli including microbial products [35]. LPS and bacterial DNA or CpG-ODN-induced activation of MAPK cascades, which in turn activates NF-κB, have been well characterized in cells of mammalian immune system [24]. In avian macrophage cell line, we have demonstrated here that activation of MAPK cascade is essential for both CpG-ODN- and LPS-mediated NO induction (Fig. 4). Cellular signalling cascades are complex interconnecting networks and are often redundant. Our results clearly show that more than one signal pathway simultaneously participates in the signal transduction by which LPS and CpG-ODN stimulate the HD11 cell activation and NO production.

Our study demonstrated that both CpG-ODN and LPS induce iNOS to generate significant amount of NO in HD11 cells and their stimulatory activities require activation of transcription factor NF-κB. NF-κB plays a central role in inflammation by regulating expression of genes that encode pro-inflammatory cytokines and chemokines and inducible enzymes such as iNOS in mammalian immune cells [55]. Extracellular stimuli such as cytokine and microbial molecules utilize divergent signalling pathways that converge on the activation of NF-κB by phosphorylating the inhibitory protein IκB. An inhibitor that blocks the IκB phosphorylation can prevent the activation of NF-κB. Both LPS and CpG-ODN responded similarly to the inhibitor BAY 11-7082 that inhibits phosphorylation of IκB, resulting in dose-dependent reduction of NO production (Fig. 5). High concentration of BAY 11-7082 in the media completely abolished both CpG-ODN- and LPS-induced NO production. These results confirm the essential role of NF-κB in the activation of macrophage HD11 by CpG-ODN and LPS.

In summary, our study has demonstrated, using an avian macrophage cell line and pharmacological inhibitors, that: (1) CpG-ODN cellular uptake is mediated by clathrin-dependent endocytosis and is different from LPS; (2) endosomal maturation is required for CpG-ODN-induced NO production, but not for LPS; (3) PKCα activity is essential for NO induction by both CpG-ODN and LPS; (4) p38MAPK and MEK1/2 play significant roles in both CpG-ODN- and LPS-induced NO production; (6) activation

of NF-κB is the key signal step in both CpG-ODN- and LPS-mediated NO induction; and finally (7), CpG-ODN and LPS stimulate iNOS to produce NO in HD11 cells.

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